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***In vivo* screening of modified siRNAs for non-specific antiviral effect in a small fish model: number and localization in the strands are important**

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ABSTRACT

Small interfering RNAs (siRNAs) are promising new active compounds in gene medicine but the induction of non-specific immune responses following their delivery continues to be a serious problem. With the purpose of avoiding such effects chemically modified siRNAs are tested in screening assay but often only examining the expression of specific immunologically relevant genes in selected cell populations typically blood cells from treated animals or humans. Assays using a relevant physiological state in biological models as read-out are not common. Here we use a fish model where the innate antiviral effect of siRNAs is functionally monitored as reduced mortality in challenge studies involving an interferon sensitive virus. Modifications with locked nucleic acid (LNA), altritol nucleic acid (ANA) and hexitol nucleic acid (HNA) reduced the antiviral protection in this model indicative of altered immunogenicity. For LNA modified siRNAs, the number and localization of modifications in the single strands was found to be important and a correlation between antiviral protection and the thermal stability of siRNAs was found. The previously published sisiRNA will in some sequences, but not all, increase the antiviral effect of siRNAs. The applied fish model represents a potent tool for conducting fast but statistically and scientifically relevant evaluations of chemically optimized siRNAs with respect to non-specific antiviral effects *in vivo*.

INTRODUCTION

Small interfering RNAs (siRNAs) program specific gene transcripts for destruction via the cellular RNA interference pathway (1). SiRNAs typically consist of two 21-nt long strands annealed to generate 19 bp with a 2-nt overhang at each 3'-end (2,3). Upon delivery to the cell cytoplasm, siRNA duplexes are taken up by the RNA induced silencing complex (RISC) (4–6) in which the Argonaute 2 component, cleaves one strand by its endonuclease activity leaving the antisense strand (AS) to bind and induce cleavage of the complementary mRNA transcripts (7–10). As this enables down-regulation of specific genes (11) siRNAs are both regarded as valuable tools in functional genomics as well as potential therapeutic agents (12). However, the occurrence of cellular immune receptors capable of recognizing single and double-stranded RNA species (13–22) complicates the interpretation of siRNA experiments (23–25) as these receptors activate innate immune defence mechanisms which can potentially lead to physiological changes of the biological system under investigation (26–28). Chemical modifications of siRNA duplexes have been shown to affect the regulation of genes involved in innate immunological response using either cell cultures or specific cell types from treated or non-treated mice or human blood cells typically peripheral blood mononuclear cells (PBMC's) or levels of immunologically relevant proteins in serum (18,29–37) but still little is known about the functional effects and consequences of such regulation in animal model assays where chemically modified siRNAs are applied. In this study, we test a range of modified siRNAs for their ability to induce a non-specific antiviral response in a small

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vertebrate model. We exploit our previous finding that the activation of a functional innate immune mechanisms by delivered siRNAs can delay and reduce the mortality of small juvenile rainbow trout challenged with the fish pathogenic rhabdovirus *Viral Haemorrhagic Septicaemia Virus* (VHSV) (27). Our data indicate that the induced immune effect of modified siRNAs *in vivo* is determined by several factors such as nucleotide sequence of the siRNA, modification chemistries, number and localization of modifications and probably, as our results seem to indicate, the thermal stability of RNA bindings.

MATERIALS AND METHODS

Synthesis, purification and annealing of duplexes

The synthesis of non-modified and chemically modified siRNAs was performed as described in Bramsen *et al.* (38,39) using previously published sequences designed to target the reporter gene enhanced green fluorescent protein (EGFP) and the housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from mouse (39; appendix 2). Locked nucleic acid (LNA; 40) modified oligonucleotides were obtained by using commercially available LNA phosphoramidite building blocks (www.exiqon.com). The chemical synthesis of the remaining modified phosphoramidites have previously been described: HM (4'-C-hydroxymethyl-DNA) (41), OMe (2'-O-methyl) (41), ANA (altritol nucleic acid) (42), HNA (hexitol nucleic acid) (43) and AEM (2'-aminoethoxymethyl) (44). Their structures are shown in Figure 1. Following annealing of strands the formation of double-stranded siRNAs was verified by 12% PAGE.

In vivo screening for the antiviral potential of duplexes

Duplexes were formulated in the liposome-formulated polycationic transfection agent 1, 2-dioleoyl-3-trimethylammonium propane (DOTAP; Roche Diagnostics, Basel, Switzerland), injected into the intraperitoneum (IP) of 1g large rainbow trout followed by challenge

with VHSV (27). Briefly, 1 µg siRNA was mixed with 2 µg DOTAP in 0.9% NaCl (physiological saline, Nycomed, Denmark). Fish were anaesthetized in 0.01% benzocaine (ethyl *p*-aminobenzoate, Sigma, Brøndby, Denmark) and injected intraperitoneally with 20 µl of the formulated siRNAs using a 27 G needled syringe before adding them to the respective aquaria's containing 8 l of running tap water. A total of 2 × 30 fish were included in each group. The water flow was stopped 24 h post-siRNA injection followed by addition of VHSV isolate DK-3592B (45) to each of the experimental aquaria giving an approximate titre of 10⁵ TCID₅₀/ml in the water of each aquarium. After 2 h of exposure the water flow was restarted. For the next 2 weeks, diseased fish were daily registered and removed from each aquarium, examined for external signs of the disease and frozen down for later verification of VHSV infection. Verification was carried out by testing material of homogenized whole fish (without tail fin) by a VHSV specific ELISA (46). Relative percent survival (RPS) at Day 10–12 post-viral challenge was calculated from the established mortality curves (Figures 2, 3 and 4) as RPS (%) = 100*[1 – (mortality fish / mortality positive control fish)]. The respective time points post-challenge for calculating RPS were chosen because they corresponded with nearly full mortality of non-treated positive control fish challenged with virus as well as with near linearity of the rest of the mortality curves.

Estimating the binding energy of annealed duplexes

The thermal stability of annealed siRNA duplexes was estimated by analysis of dissociation curves generated in a Stratagene Mx3000p RealTime PCR machine as has been described for annealed strands of DNA and RNA (47). Briefly 5 µl of the duplex dilution was added to a mixture containing 19 µl physiological saline (0.9% NaCl) and 1 µl SYBR Green (Molecular Probes, Invitrogen, Denmark) pre-diluted 1:1000. A dissociation curve was recorded while increasing the temperature from 25°C to 95°C at a temperature change of 1.8°C/min.

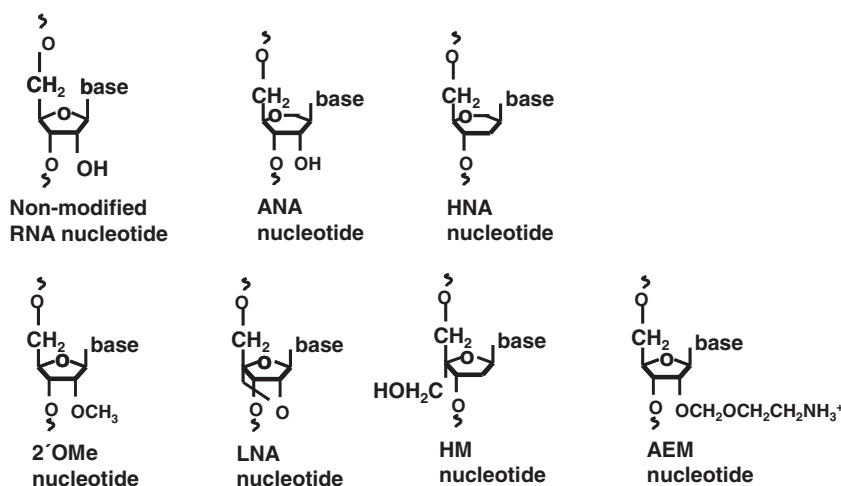


Figure 1. Chemical structures of the oligonucleotides used to modify siRNA strands in this study. See text for explanation of acronyms. RNA, ANA, HNA, 2'-OMe, LNA, HM and AEM.

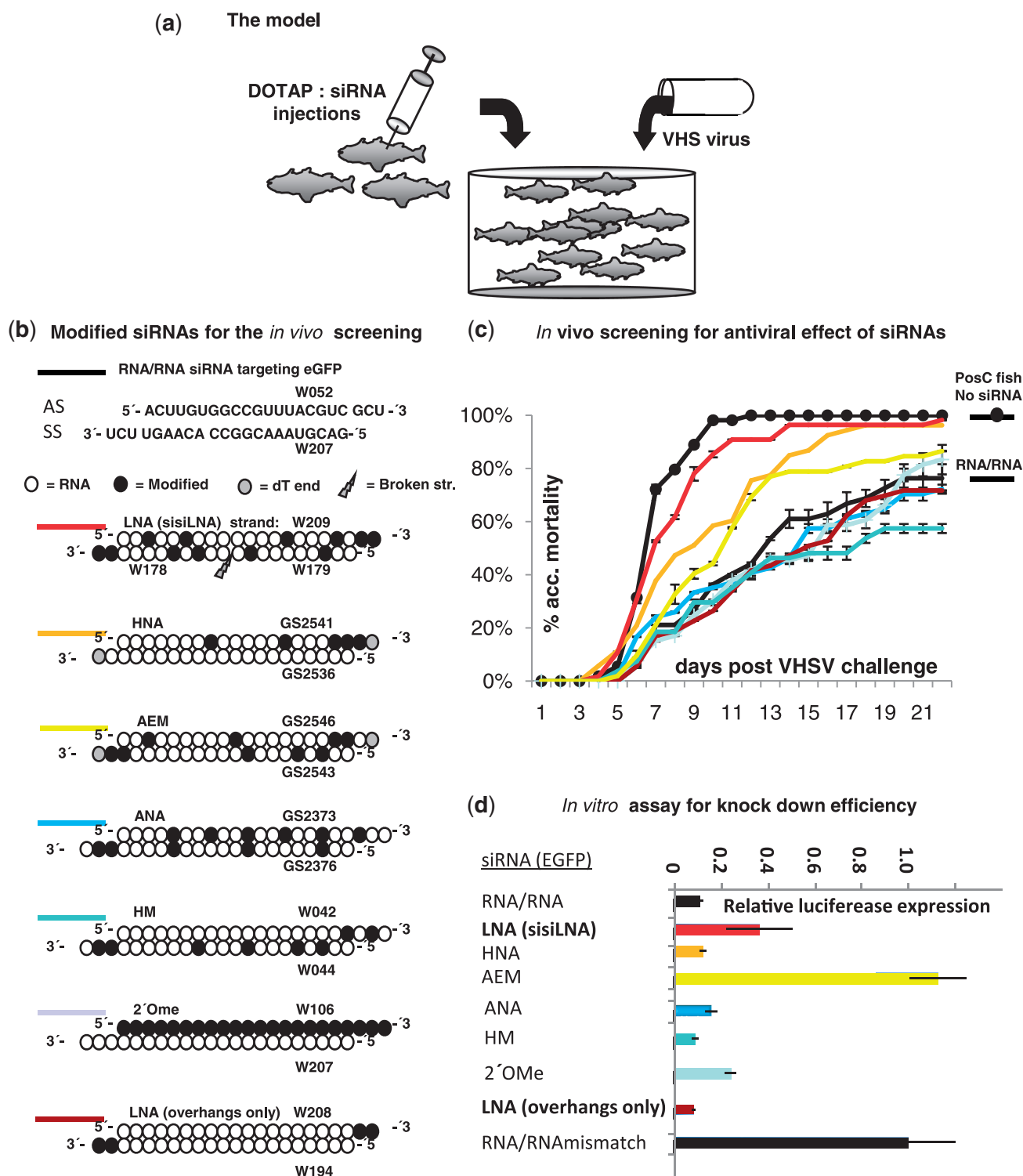
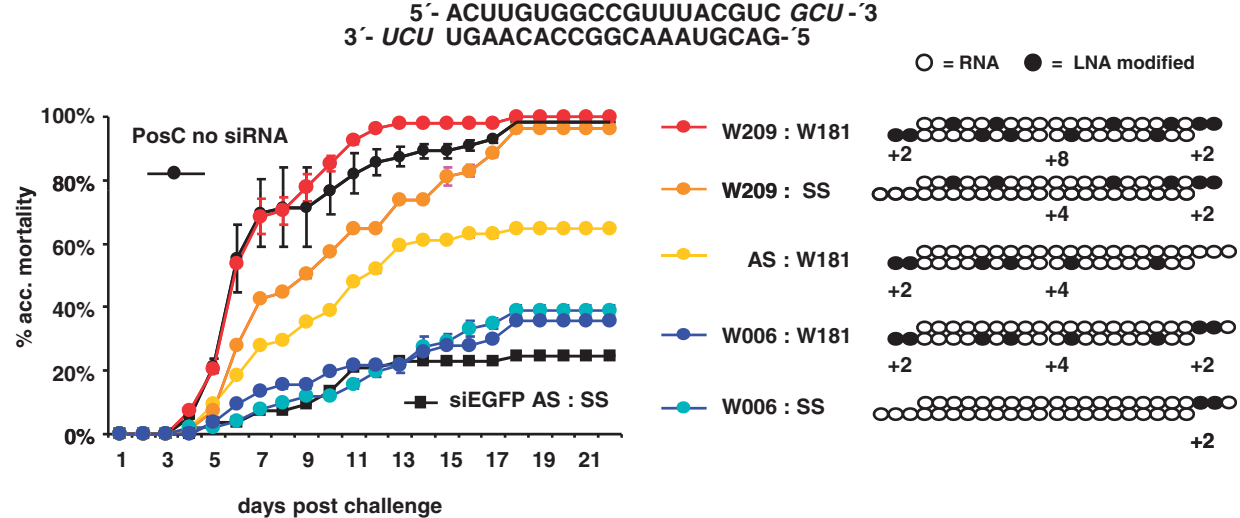


Figure 2. The fish-virus model is able to distinguish between the levels of antiviral protection induced by chemically modified siRNAs. Variants of one siRNA targeting the VHS virus irrelevant reporter gene EGFP were formulated in DOTAP and IP injected into small rainbow trout prior to challenge with the fish pathogenic and interferon sensitive VHS virus (a). Sequences and modifications of strands used to generate the injected siRNAs can be seen in Supplementary Table S1, but are shown here diagrammatically (b). All strands were chemically modified variants of the siRNA sequence seen in the upper panel. Strand numbers used throughout the text are placed next to the diagrammatic representations of strands. The chemistries used to modify RNA strands are shown in Figure 1 (the meaning of white, grey and black ball shapes as well as the lightning symbol is explained in the diagram). In order to generate mortality curves (c) the dead fish from the challenge study were counted and monitored as mean accumulated mortality \pm SD (error bars) of treated groups. Each group was run in duplicate with 30 fish per replicate. (d) Knock-down activity of the modified siRNAs was examined by the use of an *in vitro* luciferase assay as explained in 'Materials and Methods' section. The colour coding used for the various modified siRNAs in (b) is equal to the one used in the mortality curves (c) and in the figure on the *in vitro* knock-down effect (d).

(a) EGFP targeting siRNA



(b) GAPDH targeting siRNA

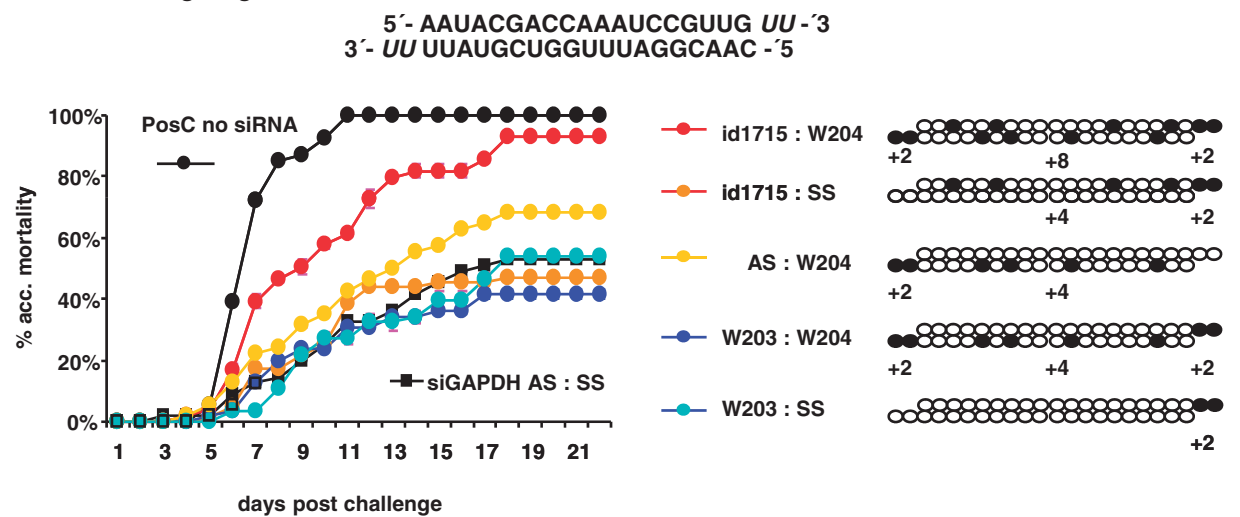


Figure 3. The number and localization of LNA modifications in the siRNAs seems to determine their antiviral effect in the fish-virus model. Screening of LNA modified variants of one siRNA-targeting EGFP (a) and of one targeting GAPDH (b) using the same pattern of modification showed that there is also an effect of sequence. Right panel: diagrammatic representations of siRNAs with white ball shapes as RNA nucleotides and black ball shapes as LNA. Counts below the siRNA diagrams are counts of LNA modifications inside the duplex part and in the overhangs respectively. Challenge experiments and mortality curves were generated like explained for Figure 2. Colour codes were used to differentiate between siRNAs. Detailed sequence information with annotation of modified bases can be found in Supplementary Table S2.

Emitted fluorescence from SYBR Green bound to duplexes was recorded every 0.5s. The FAM filter set with excitation wavelength at 492 nm and emission wavelength at 516 nm was used. MxPro software (Stratagene, La Jolla, CA) was used for determining T_m by the $-R'(T)$ methods, where T_m is defined as the temperature where the drop in fluorescence is maximal. As melting is performed relatively fast we cannot expect equilibrium between bound and unbound strands to be reached during measurement which is why we do not regard measured melting temperatures to be exact values but rather relative approximations of thermal stability between differently modified siRNAs. The approximated

thermal stabilities of siRNAs were correlated with the RPS of fish treated with these siRNAs in the challenge studies (see 'Materials and Methods' section above on the *in vivo* screening of antiviral potential of duplexes).

TNF α regulation in human PBMC treated with siRNAs

PBMCs from healthy volunteers (200 000/well) were treated with 100 nM siRNA-targeting EGFP mRNA, again using DOTAP as the delivery reagent. After 9, 12 and 18 h the supernatant from the cells was collected and frozen in -80°C until use. The samples were then assayed on human TNF- α ELISA MaxTM Deluxe Sets (Biolegend #430205).

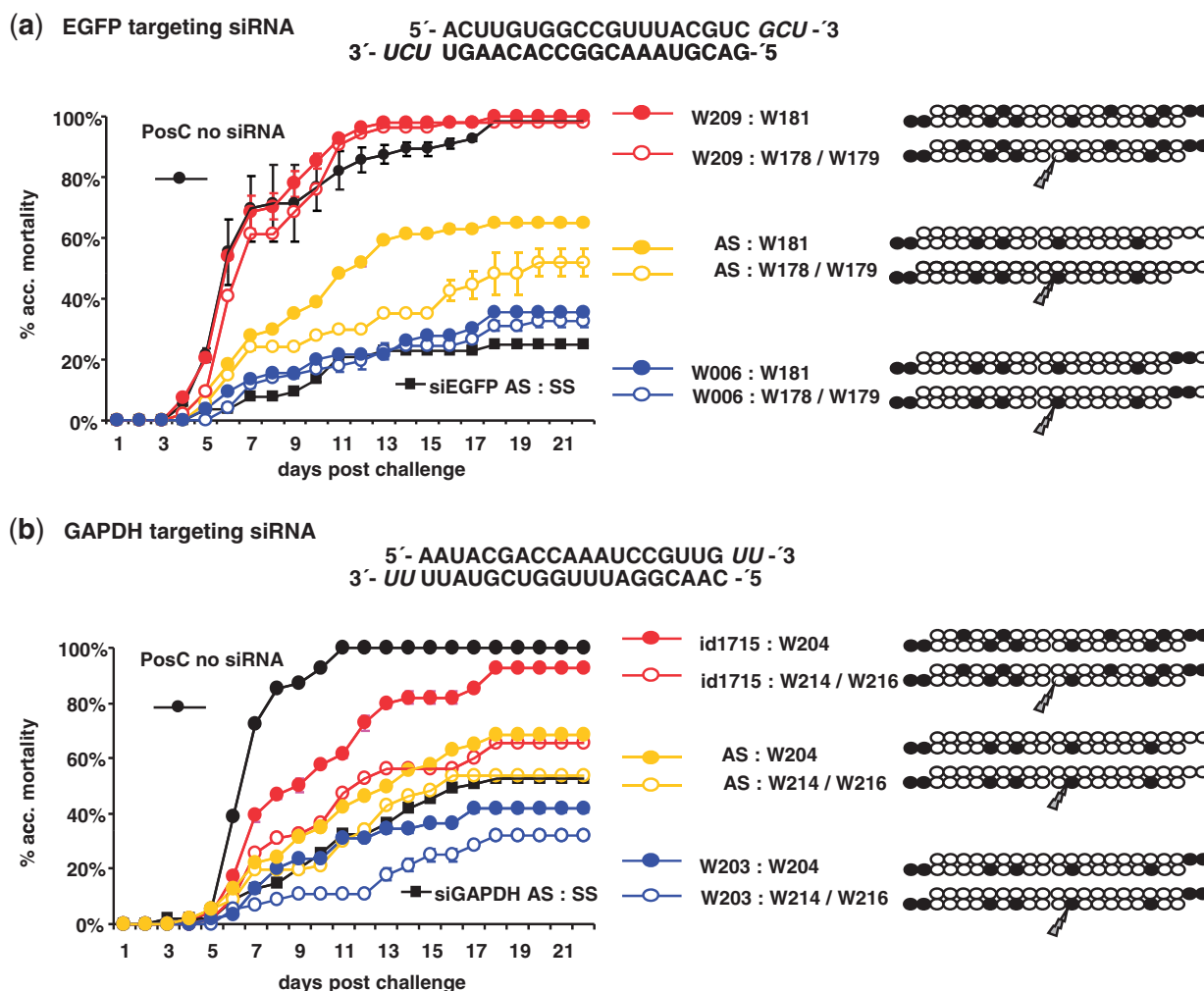


Figure 4. A nick introduced in the SS increases antiviral effect in the fish-virus model. **(a)** Screening of the LNA modified siRNAs W209:W181, AS:W181 and W006:W181 (from Figure 3a) against versions of the same siRNAs where the W181 strand was substituted with the two smaller strands W178 and W179. **(b)** In the same way the LNA modified siRNAs from Figure 3b: id1715:W204, AS:204 and W203:W204 were compared to siRNAs where W204 had been substituted with the two strands W214 and W216. Challenge experiments and mortality curves were generated using two duplicate aquaria of 30 fish per treatment group. Mean mortalities \pm SD. Colour codes were used to differentiate between siRNAs. Sequence information for these siRNA strands can be found in Supplementary Table S2. A nick in the SS is noted by a lightning symbol in the diagrammatic representation of strands.

Test of RNAi functionality of modified siRNAs *in vitro*

Test for siRNA silencing activity was done using a previously established protocol (48). Briefly, the human lung cancer cell line H1299 was grown in RPMI-1640 containing 10% FBS, 1% penicillin/streptomycin. A stable luciferase reporter H1299 cell line was created by transfection with a psiCHECK2 vector (Promega) modified to contain a perfect target site for the EGFP specific siRNA in the multiple cloning site (MCS) located in the 3'-UTR of the Renilla luciferase gene. A puromycin resistance cassette in the vector allowed for the selection of stably transfected cells (1 μ g puromycin/ml medium). Evaluation of knock-down efficiency was performed using this cell line and transfections with EGFP specific siRNAs at 10 nM concentrations in triplicates. Lipofectamine 2000 reagent (Invitrogen) was used in a 96-well format with 15000 cells in 100 μ l serum-free

RPMI per well. After 4 h, the transfection mixture was substituted with growth medium containing serum. Cell lysates were harvested after 48 h and luciferase assays were performed using the 'Dual-luciferase reporter assay system' (Promega) according to the manufacturer's protocol on a FLUOstar luminometer (BMG labtech); Renilla luciferase signals (sample) were normalized to the firefly luciferase signals (transfection control).

RESULTS

Chemical modifications of siRNAs modulate their antiviral effects in a viral challenge model

In the VHS virus challenge model, rainbow trout injected with a non-modified siRNA targeting the virus-irrelevant reporter gene EGFP (RNA/RNA-targeting eGFP duplex in Figure 2) experienced a lower degree of virus induced

mortality compared to buffer injected control fish (PosC no siRNA in Figure 2c). Initial experiments using 1–2 µg siRNA did not show signs of concentration dependence upon mortality why we are probably working with a saturated system (data not shown). We have previously shown indirect evidence that the non-specific reduction of mortality was due to induction of an interferon response (27). In order to determine whether chemical modification could influence the antiviral effect, we screened a range of modified versions of this siRNA (Figure 2b) for their ability to protect fish from viral induced mortality (Figure 2c). Most of the modified siRNAs induced a high antiviral protection equal to or in some cases even stronger than that induced by the non-modified siRNA indicative of a potent interferon response. However, three of the modified siRNAs, containing LNA, HNA or AEM nucleotides respectively exhibited a reduced antiviral effect as revealed by an increased and more acute mortality in the challenge model (lighter red, orange and yellow curves in Figure 2c). Especially the sisiRNA (40), which was LNA modified in the stem part and where the passenger strand [sense strand (SS)] was replaced by two shorter 10-nt and 12-nt strands, showed a dramatically reduced antiviral effect compared to the non modified RNA/RNA duplex. In contrast, the siRNA which was LNA modified only in the overhangs (W208:W194), showed no abrogation of antiviral response in our model. Except from the AEM modified version, all the siRNAs retained knock-down functionality *in vitro* (Figure 2d), although the sisiRNA seemed less potent. Accordingly, our initial observations show that it is possible to strongly reduce induction of innate mechanisms while still retaining a significant knock-down effect. Interestingly we did find a strong antiviral effect of our 2'OMe nucleotide modified siRNA despite that 2'OMe has previously been shown to be a potent antagonist of immunostimulatory RNA in the mammalian systems where it was tested (32,35).

LNA modifications in the siRNA stem reduces antiviral effect

The effect of introducing LNAs into siRNAs was investigated by varying the number and positions of modifications in the EGFP-targeting siRNA (Figure 3a). In order to control for sequence related effects we also included non-modified and modified versions of a previously published GAPDH-targeting siRNA using the same modification patterns as for the EGFP-targeting siRNA (Figure 3b). The setup included native strands, strands with four LNAs in the stem and two LNAs in the overhang and finally strands with only two LNAs in the overhang (only AS). Pairing of AS and SS containing six LNAs in both strands strongly reduced the antiviral effect of both the EGFP and the GAPDH specific siRNA seen as a higher mortality compared to the non-modified siRNAs (compare the siRNAs W209:W181 and id1715:W204 with the non-modified siRNAs called AS:SS in Figure 3a and b respectively). When shifting strands to ones containing a lower number of LNA residues in the stem part of siRNAs these again resumed

their antiviral induction to different levels seen as varying degrees of reduction in the mortality curve. Duplexes containing an AS strand with only 3'-end LNA modification (W006:W181, W006:SS and W203:W204, W203:SS in Figure 3a–b) showed no abrogation of antiviral response compared to the siRNA composed only of RNA. This was also seen when LNA substitutions were placed in both overhangs (W208:W194 in Figure 2) and did not seem to be related to whether the overhang was 3- or 2-nt long.

A knick in the SS (sisiRNA) increases the antiviral effect in some siRNAs

As LNA nucleotide substitutions are known to increase the thermal stability of RNA and DNA duplexes (40,49) we checked whether a reduction of duplex stability through introduction of a destabilizing break in the passenger strands would be able to increase their antiviral effect (Figure 4a and b). This was the case, but the effect was most pronounced for the GAPDH siRNA where the sisiRNA design led to decreased mortality for all modification patterns.

A high LNA load reduces knock-down efficiency but can be partly restored by the sisiRNA design

We checked the knock-down efficiency of our siRNAs in an *in vitro* assay using human cells and found that all worked as potent inhibitors except for the highly LNA modified W209:W181 double-strand (Figure 5). On the other hand, the sisiRNA design of this duplex (W209:W178/W179) increased the knock-down efficiency from 0% to 65%, which shows that we can, as previously postulated, increase the knock-down efficiency of these modified siRNAs by pre-cleaving the passenger strand (39). The interesting finding here was that whereas this cleaving in many cases resulted in higher antiviral effect (lower mortality in the challenge study) such an effect was not significant for the W209:W178/W179 duplex (Figure 4a) indicating that it might be possible in some cases to design LNA modified siRNAs causing none or only insignificant side effects while still retaining their knock-down efficiency.

Discrepancies between the fish model and a mammalian *in vitro* system

We investigated whether the innate immune response to the siRNA variants, as observed in the fish model, could be recapitulated in human PBMCs using the level of the proinflammatory molecule TNF-α as read-out (Supplementary Figure S1). We saw the tendency of decreased immune stimulation with increased LNA load, but whereas the immune stimulatory effect of siRNAs containing LNA modifications in the overhang only (siRNA harbouring the W006 strand) resembled that of the unmodified RNA duplex in the fish model this was not as clear in the human PBMC's where only the W006:SS duplex was highly stimulatory.



Figure 5. Highly LNA modified siRNAs show low knock-down efficiency which is possible to regain by introducing a nick in the SS. Such findings have previously been shown (29) but we tested it again on the specific strands used in this study. The cell culture assay is described in 'Materials and Methods' section. Data are mean values for triplicate wells and error bars represent SDs. All values were normalized to the mean signal from cells treated with a mismatched siRNA not expected to target the luciferase expression. An expression value of 1 is equal to no target reduction (100% luciferase signal). The strand numbers in the left panel are the same as used throughout the text and Figures 3 and 4.

Antiviral effects of LNA modified siRNAs in the fish model are correlated with their thermal stability

The suggested relationship between duplex stability and immune stimulatory properties was further analysed by plotting estimated melting temperatures of siRNAs with their corresponding effect on mortality in the infection trials. For LNA modified siRNAs (presented in Figures 3 and 4) we found a positive correlation between the relative thermal stabilities of LNA modified siRNAs and their antiviral effect seen in our infection model (Figure 6b–c). Such a relationship should probably not be expected for all types of modifications as indicated by our initial screening experiments where thermal stability could not explain differences in immunostimulation across different types of modifications (Figure 6a; siRNAs from screening shown in Figure 2).

LNAs in the siRNA stem can abrogate the effect of a previously published immune stimulating motif

To investigate the generality of our observations in the fish model, we tested an siRNA previously found to contain a mammalian immune stimulatory motif ('GUCCUU CAA') in the SS and compared this to a modified siRNA containing four LNA substitutions inside the motif as a means of abrogating the stimulatory effect (18). Using our *in vivo* model, we found that four LNA-modifications within the immune stimulatory motif reduced the antiviral effects significantly (Figure 7a) in agreement with the mammalian study (18). Notably, a similar reduction in antiviral effect was seen when the LNA substitutions were placed in the AS of the region complementary to the immune stimulatory motif. For these siRNAs melting temperature and %RPS was also negatively correlated (Figure 7b). In the human PBMCs

using TNF- α as a measurement of immune induction we found nearly similar results (Supplementary Figure S2).

Single strands induce the antiviral response which can be abrogated by LNA modification

We tested whether the single strands used to generate our siRNA duplexes could themselves induce the antiviral response in the fish model as has previously been shown in mammalian cells (18,21,33). This was also the case in the fish model where our results indicated that the antiviral effect of the duplexes could be explained by the effect of the strongest inducer of the two strands [Figure 8a–b; AS from the AS:SS (non-modified RNA duplex), W006 from W006:W181 and SS from W006:SS]. This indicated strand competition for receptors rather than an additive effect of strands although this should be further investigated by using a larger set of sequences. The same was seen when using two strands of the same orientation which are not expected to hybridize (Figure 8c; Combinations of the AS, which is the non-modified RNA AS, the W006 strand which was only end modified by LNA and the W010 strand containing six LNA modifications).

DISCUSSION

Viral diseases of rainbow trout are well studied due to the use of this species in aquaculture. Good infection models exist making this an interesting animal model for the study of antiviral medicines. Recently, the use of siRNA knock down for targeting viral diseases has been tested in rainbow trout (27), but given the strong non-specific antiviral effect, which are under some circumstances induced by siRNAs, it can, like in the mammalian studies (23), be difficult to get a relevant read-out of the specific effect (26,27). In rainbow trout we have previously shown that IP injected siRNAs, when formulated by the effective liposomal delivery reagent DOTAP, are first taken up by macrophage-like cells in the intraperitoneum followed by an antiviral immune response which is able to protect the fish against VHS virus induced mortality (27). Previously IP injected mice were shown to take up DOTAP formulated siRNAs by the same route, but unfortunately the absence of an interferon response in this model was only studied in intravenously (IV) injected mice (50). It has previously been reported that introduction of chemical modifications into siRNAs were able to reduce their induction of innate immune mechanisms in mammalian systems (17,18,22,23,28–35). Here we used the fish-virus model to screen for types of modifications which reduce the ability of siRNAs to act as antiviral stimulants *in vivo* without compromising their knock-down efficiency as evaluated *in vitro* in a mammalian system. The initial screenings clearly demonstrated that the fish-virus model could differentiate between different modified versions of the siRNA based on their antiviral effect towards the VHS virus. Notably, the antiviral effect of the LNA modified sisRNA, the HNA and the AEM modified siRNAs were significantly reduced compared to the effect seen when using the non-modified siRNA (Figure 2c), but only the

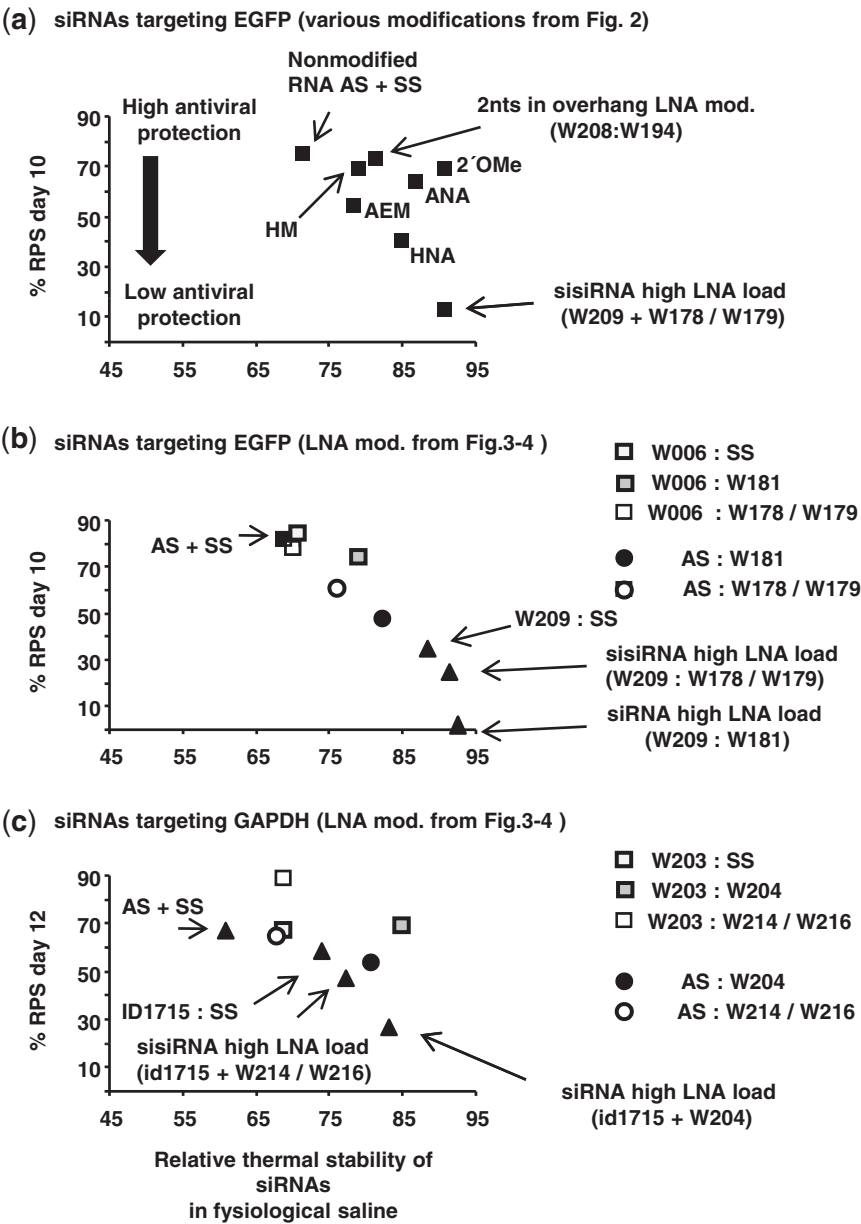


Figure 6. Antiviral effect of siRNAs in the fish-virus model showed a negative correlation with the estimated thermal stabilities for the LNA modified siRNAs (b–c) but not for the assembly of modified siRNAs screened in Figure 2 (a). RPS was calculated from the mortality curves shown in Figure 2 (6a) and 3–4 (6b–c) respectively using the procedure described in ‘Materials and Methods’ section and correlated with relative thermal stability of siRNAs estimated as the melting temperature found by dissociation curve analysis. Strand and modification names refer to the ones used in Figures 1, 2, 3, 4 and the Supplementary Tables S1 and S2.

LNA modified sisiRNA and the HNA modified siRNA without abrogating the knock-down potential (Figure 2d). We further investigated the role of LNA modification. Our results indicated the importance of the modification pattern in our siRNAs. Introduction of LNAs inside the stem part generally reduced the antiviral stimulation in the fish as compared to modifications in the overhangs. A role of LNA modifications in siRNA overhangs on cellular stress has previously only been described with respect to reduced viability in HeLa cells (38; Here using the same strands W006 and W203 as we used in our study). This could potentially be due to the innate response we saw as a functional antiviral protection in our model. But

we were not able to show the same general effect of including the W006 strand in our human PBMC cell culture experiments where only the W006:SS siRNA strongly induced the expression of TNF α (Supplementary Figure S1). Discrepancies between the results from the mammalian *in vitro* models including our own and our fish *in vivo* model systems could result from the former using human PBMC's in *in vitro* systems and only assaying the regulation of a specific stress or immunologically relevant gene. One other interesting difference between the innate response in our model and previous mammalian models included the absence of an effect by our 2'-OMe modified siRNA. As we only included one

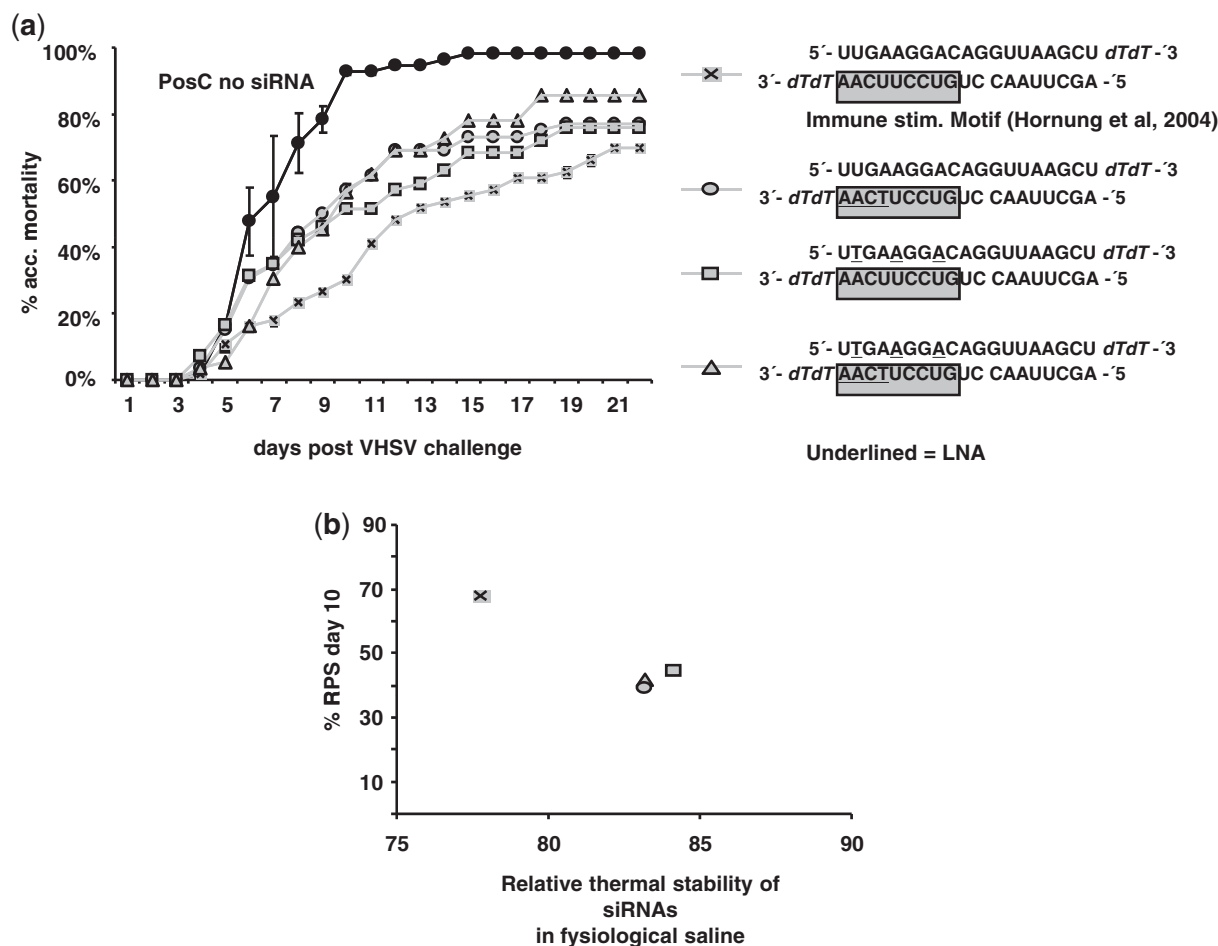


Figure 7. The relation between thermal stability and antiviral effect *in vivo* was also found using LNA modifications of a previously published siRNA containing an immune stimulatory motif (13). Challenge experiments (a) and estimation of siRNA thermal stability was carried out as described in the text. The immune stimulating motif is boxed in grey in the sequence diagrams in (a). The first siRNA from the top is the non-modified siRNA. The second from the top is also a sequence from the Hornung *et al.* study where the immune stimulatory effect of the siRNA was reduced by substituting four RNA nucleotides with LNA nucleotides in the motif (LNA residues are underlined). The next siRNA was our own design and included LNA modifications only in the strand opposite to the proposed motif. In the last siRNA (lower row) modifications were introduced into both strands. (b) We show that RPS and thermal stability of these siRNAs are negatively correlated. Methods are as described in the “Materials and Methods” section as well as in the text for Figure 5. Mortality curves were made from mean values for duplicate groups of fish with the same treatment. Error bars represent SDs of duplicates.

2'-OMe modified duplex in our initial experiments we cannot safely say whether this is a general finding in the fish model or whether a different pattern of modification would have behaved different.

LNA modifications in the stem part of double-stranded nucleic acids are known to increase their thermal stability which could change the siRNAs ability to react with cellular receptors. For our *in vivo* model we found indications that the thermal stability of LNA modified siRNAs could be a determinant of the degree of antiviral effect induced. First, we demonstrated that fish injected with siRNAs containing more LNA modifications in the stem experienced a higher mortality following viral infection indicative of a lower innate antiviral stimulation. Secondly, the introduction of destabilizing nicks in the stem part (the sisiRNA design) had the opposite effect. Thirdly, the relative thermal stability between strands as estimated by the dissociation curve method (47) was negatively correlated with the RPS in the viral challenge studies.

Furthermore, we found a correlation between thermal stability and antiviral effect of a previously published siRNAs harbouring an immunostimulatory motif (18).

One possible explanation for the influence of thermal stability of siRNAs upon antiviral activation when delivered by DOTAP comes from recent findings that this cationic liposome based delivery reagent promotes efficient delivery via uptake into endosome compartments of the vertebrate cell (21,51). Here they allow for the recognition of both double-stranded RNA and single-stranded RNA species by TLR receptors 3, 7 and 8 all signalling the initiation of the interferon response (19; 51). At least in isolated mouse leucocytes the single strands of siRNAs have been shown to act as ligand for TLR-7 and 8 (18). This could explain the correlation between thermodynamic stability and antiviral effect in our *in vivo* model. Importantly, fish also have TLR3, 7 and 8 although two forms of TLR8 are present in the rainbow trout (52–54).

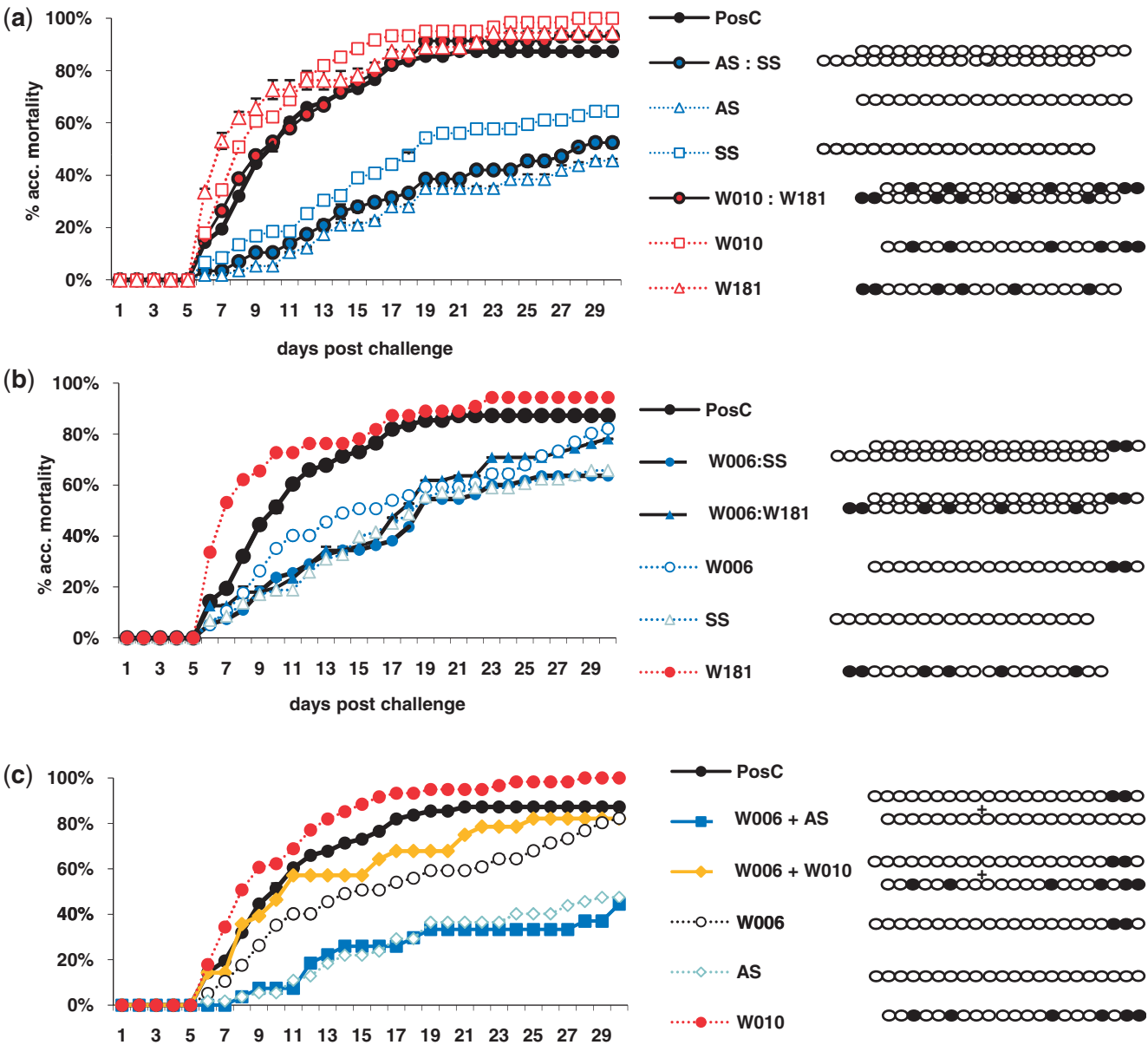


Figure 8. LNA modification is able to reduce the antiviral effect induced by single RNA strands in the fish model. The antiviral effect of the single strands seems to determine the effect of the corresponding siRNA duplex in a non-additive manner. Strands screened were the AS, SS, W010, W181 and W006 (siRNA-targeting EGFP; Figures 3a, 4a and Supplementary Figure S2) including combinations of these which were formulated in DOTAP and injected into rainbow trout subsequently infected with VHSV as described in ‘Materials and Methods’ section’. Mortality curves and error bars where made as described in previous figures. The W010 strand resembles the W209 strand used throughout this study, but contains an extra U in the 3'-end. The three graphs a, b and c represents data from the same experimental set-up where 2 groups of 30 fish were used to test each strand or strand combination respectively.

In line with this hypothesis single strands were able to induce the innate mechanisms and explain the antiviral effects of siRNAs in the fish. But, our finding that LNA modification of single strands could also reduce the antiviral effect outside a duplex context shows that the effect of LNA can at least not exclusively be assigned to the strengthening of the siRNA duplex. As the number of LNAs in nucleic acid duplexes is strongly correlated with their stability, the correlation we initially found between thermal stability and antiviral effect could partly be due to a secondary correlation between lower

innate induction by LNA nucleotide chemistry on the one side and higher thermal stability introduced through LNA modification on the other side. But, this does not explain why breaking the passenger strand as in the siRNA design also increased the antiviral effect of siRNAs. Breaking one of the strands in a duplex would mean that three new strands would be responsible for increasing the antiviral effect and we have no evidence of smaller strands being better at stimulating cellular receptors for inducing an interferon response. In fact the opposite is usually proposed (17,55).

An alternative explanation is that by cleaving the strand we destroy either a motif or a secondary structure of the single-stranded RNA which could have an impact on its ability to react with cellular receptors. We speculate whether the effect of LNA modification upon the antiviral effect of siRNAs could be caused by strengthening of secondary structures in the single strands thereby making them less fit for reaction with receptors and we suggest that the stability of potential RNA structures should be investigated as one factor involved in determining the strength of the immune response induced by siRNAs. It should be mentioned here that a correlation between the thermal-binding stability between the two strands of non-modified siRNAs and their antiviral effect when formulated in DOTAP has previously been described for human PBMC's *in vitro* (56). It is also important to note that a possible relationship between thermal stability and antiviral effect should not be expected to hold true for all types of modifications as there may be differences in the immune stimulatory potential of different chemistries as also evidenced by our results in the first screening (Figure 2).

A quite different possibility why increasing the LNA content of siRNAs could render them less immunestimulatory is related to the number of uracil residues which is one factor which has been suggested to be of importance for immune induction (56). The most antiviral of our two non-modified siRNAs (the GAPDH-targeting duplex) contained 15 uracils compared to the less antiviral EGFP-targeting duplex containing 12 uracil residues (Figure 3 and Supplementary Table S2). This relationship was also seen for the single strands as the AS of the EGFP-targeting siRNA (containing eight uracil residues) was more immune stimulating than the RNA SS containing only four uracils residues (Figure 8a). The number of uracils in our siRNAs changed when RNA uracils were substituted with LNA thymidines. This could have been the reason for the sequence specific LNA induced changes in innate immune induction we saw even when using the same pattern of LNA substitutions because the two different sequences contained different number and patterns of uracil bases. There was indeed some evidence in our results that LNA based changes of U's to T's could have had influence on reducing the antiviral effect. As an example this could explain the reduction in antiviral protection in fish injected with the highly LNA modified siRNA's W209:W181 (number of U's reduced to 5 compared to the non-modified EGFP-targeting siRNA containing 12 U's; Figure 3a) and ID1715:W204 (number of U's reduced to 8 compared to 15 U's in the non-modified siRNA-targeting GAPDH; Figure 3b) and it also gives us an explanation on why the second of these sisiRNA's was more immune stimulating than the first. But this could not explain the increase in the antiviral effect upon breakage of the passenger strand as seen for the sisiRNA design.

While our study and a some previous studies have shown that LNA substitutions could reduce toxicity (38) and antiviral effects (18) of siRNAs we have also show that a high load of LNA substitutions reduces siRNA efficiency. A nick in the passenger strand is able to partly

rescue knock-down efficiency of such highly LNA modified siRNAs [Figure 5 and (39)] for some siRNA sequences even without increasing the antiviral effect significantly (Figure 5a). The sisiRNA design could therefore be relevant in allowing us to design efficient siRNA sequences with reduced side effects.

When using *in vitro* assays for testing immune stimulation by delivered chemically modified siRNAs usually regulation of a single stress gene in a selected cell type is measured. Accordingly, the reductions seen in cellular stress or immune stimulation is seldom related to pathologies or physiological stress disturbing the system of interest. In this study we use a test where modified siRNAs are screened in a biological system where a given advantageous method of delivery induces a strong cellular stress disturbing a potential specific read-out and imposing side-effect on the fish (inflammation in the fish was strong causing muscle haemorrhages). A different example of such a model for the non-specific effect of siRNAs was shown in a recent study of innate response induced by siRNAs through TLR3 activation which was shown to inhibit blood and lymphatic vessel growth in the eyes of mice (28). It was therefore difficult to ascertain the specific effect of siRNAs used to target the vascular endothelial growth factor-A (VEGFA) in this model (17), but it makes the model relevant for testing chemical modifications in a specific relevant system. Small animal models make it possible to increase the statistic reliability of assays through including several animals. In our experiments we used two groups of 30 fish thereby making it possible to study variability between individuals.

CONCLUSION

Using an *in vivo* fish model we assay the functional innate immune stimulation by IP injected siRNAs as an antiviral response against a deadly viral disease. We find that some modification types are able to abrogate siRNA stimulation at a relevant functional level. We find both general and siRNA sequence related effects of chemical modification. We therefore conclude that optimization on the type and number of modifications as well as on their location in the strands should be done for each new siRNA sequence.

Using our model it seemed that the non-specific innate effect of LNA modified siRNAs was determined by many factors and that both nucleotide chemistry and stability caused by LNA modifications may be such determinants. Furthermore, our results on LNA modified single strands indicated that the innate antiviral effect was non-additive but dictated by the strongest inducing strand although final proof of this point would require further experiments. We found some interesting discrepancies between our *in vivo* model and the previously published mammalian models where the regulation of specific immune genes is measured on cells purified from siRNA treated animals.

Whereas specific silencing *in vitro* is reduced by heavy LNA modification, this can to some degree be circumvented by introduction of a nick in the passenger strand (sisiRNA design) and we show that this does in some cases

but not necessarily increase the innate immune induction of siRNAs *in vivo* why this could be a viable strategy for balancing the edge of the sword between potent silencing and induction of an innate immune response.

The results are useful for the future design of efficient siRNAs with low side effect and in the continuing studies of RNAs acting as immune stimulating PAMPS in vertebrates. As the basic immune components of the innate system recognizing RNAs were already developed in the fish (57–61) the basal triggers of the system in the fish may in most respects be functionally equivalent with those found in higher vertebrates. Besides its usefulness in aquaculture research our model may therefore be interesting as a small animal model for initial tests of antiviral side-effects of RNA based drugs before carrying on the testing in more expensive mammalian models.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1–2, Supplementary Figures 1–2.

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